



# Organic phase separation opens up new opportunities to interrogate the RNA-binding proteome

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## Abstract

Protein–RNA interactions regulate all aspects of RNA metabolism and are crucial to the function of catalytic ribonucleoproteins. Until recently, the available technologies to capture RNA-bound proteins have been biased toward poly(A) RNA-binding proteins (RBPs) or involve molecular labeling, limiting their application. With the advent of organic–aqueous phase separation–based methods, we now have technologies that efficiently enrich the complete suite of RBPs and enable quantification of RBP dynamics. These flexible approaches to study RBPs and their bound RNA open up new research avenues for systems-level interrogation of protein–RNA interactions.

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## Introduction

Messenger RNA (mRNA) molecules do not exist in isolation and are instead decorated by RNA-binding proteins (RBPs) that regulate every stage of their life cycle, from transcription, during translation, and through to degradation [1]. A considerable effort has been made to understand the RNA sequence and structural features regulating these interactions (reviewed in a study by Gehring et al. [2]) and how RNA-binding protein affinity and specificity is achieved (reviewed in a study by Helder et al. [3]). More recently, our expanding understanding of the myriad functions of long noncoding RNA (lncRNA) has also established the crucial functions of protein–lncRNA interactions [4]. Moreover, mutations in RBPs have been documented in neurodegenerative diseases, muscular disorders, and cancers, further underscoring the importance of protein–RNA interactions in all aspects of cellular physiology [5]. Detailed studies of individual proteins have been largely limited to canonical RBPs [6]. However, the development of techniques to catalog the cellular RNA-binding proteome (RBPome) have identified hundreds of putative novel RBPs suggesting exciting undiscovered roles of protein–RNA interactions in regulating additional cellular functions [7]. Thanks to the new development of more efficient and unbiased technologies based on organic:aqueous phase separation, the study of RNA-binding dynamics of RBPs can now be addressed, opening new opportunities to understand RBP biology from a system-wide perspective.

## Cataloging RNA-binding proteins using oligo(dT)

Early approaches for higher throughput identification of RNA-binding proteins involved *in vitro* screens using microarrays of tagged proteins or immobilized RNA baits to identify novel RNA–protein interactions [8–10]. However, the RBPs identified by these high-throughput screens may not represent RBP interactions that occur *in vivo*. In 2012, two groups independently developed a new strategy to assess eukaryotic RBPomes *in vivo* using UV irradiation to cross-link interacting RNAs and

proteins followed by extraction of protein–RNA complexes with oligo(dT) beads (RNA interactome capture; RIC) [11,12]. The RIC principle of poly(A) RNA enrichment has since then been further modified to interrogate putative *in vivo* RNA-binding domains [13–15]. RIC has established itself as a mainstay for the study of RNA-binding proteins and has been used to catalog RBPs in many eukaryotic systems, including *Homo sapiens* cell lines and macrophages, *Mus Musculus* embryonic stem cells, *Saccharomyces cerevisiae*, *Danio rerio* and *Drosophila melanogaster* embryos, *Arabidopsis thaliana* seedlings, *Caenorhabditis elegans*, *Plasmodium falciparum*, *Leishmania donovani* and *Trypanosoma brucei* [16–25]. These studies have unearthed a multitude of novel RBPs without canonical RNA-binding domains or known links to RNA biology, hinting at undiscovered interplay between RNA metabolism and other components of cellular physiology such as cell cycle progression and metabolic fluxes [7]. Despite the success of RIC, the required starting material ( $2.8 \times 10^8$  cells [26]), restricts its application to situations where cell numbers are not limiting and constrains its use for quantifying changes in the RBPome where replicate samples and multiple conditions are required, although such studies have been performed [27]. Moreover, because RIC was designed to enrich poly(A)-binding RBPs, it is not suitable to interrogate the binding partners of nonpoly(A) RNAs such as ncRNAs, and organisms with little or no poly(A) RNA, including bacteria and many archaea. Nevertheless, the original RIC protocols were also observed to capture DNA and some nonpoly(A) RNA [28,29], complicating the interpretation of the proteins recovered. A recent modification of the RIC protocol, enhanced RIC, has helped to overcome this issue by using locked nucleic acid technology to improve the hybridization between oligo(dT) and poly(A) RNA [29]. This enables the use of more stringent wash steps to ensure efficient capture of poly(A) RNAs and reduce the background protein contamination.

### Extending into the nonpoly(A) RNA-binding proteome

There has been a recent spate of techniques using synthetic nucleotide analogs to capture proteins bound to nascent RNA, irrespective of their poly(A) status. RNA-binding region identification (RBR-ID), uses 4-thiouridine and UV 365 nm cross-linking (CL) and identifies putative RNA-binding domains based on reduced peptide intensity after CL [30]. RBR-ID relies upon a loss or decrease of signal across multiple sample workflows, which is suboptimal. Furthermore, the approach has so far only been applied to nuclei and has not been demonstrated to work with whole cell extracts. Two more promising nucleotide analog-based techniques were recently published which both use 5-ethynyl uridine combined with click chemistry to facilitate the pull-down of protein–RNA

adducts, such as RNA interactome using click chemistry (RICK) [31] and click chemistry–assisted RNA interactome capture (CARIC) [32]. Unfortunately, synthetic nucleotide analog labeling is only viable for relatively short time periods because it inhibits rRNA synthesis, causes a nucleolar stress response [33], and reduces cell viability [34]. Hence, RBR-ID, CARIC, and RICK are able to capture proteins bound to nonpoly(A) RNA but are limited to the study of proteins binding nascent RNA. Where proteins binding nascent RNA are of particular interest, such as in studies of splicing factors or proteins involved in the nascent RNA degradation, and incorporation of 5-ethynyl uridine is possible, RICK and CARIC are valuable approaches.

Additional silica-based strategies have also been developed for the complete recovery of the RBPome [35,36]. However, owing to the low recovery of RNA-bound proteins using silica [37], this approach has only been applied to large-scale liquid cultures of *S. cerevisiae* and *Escherichia coli* [36].

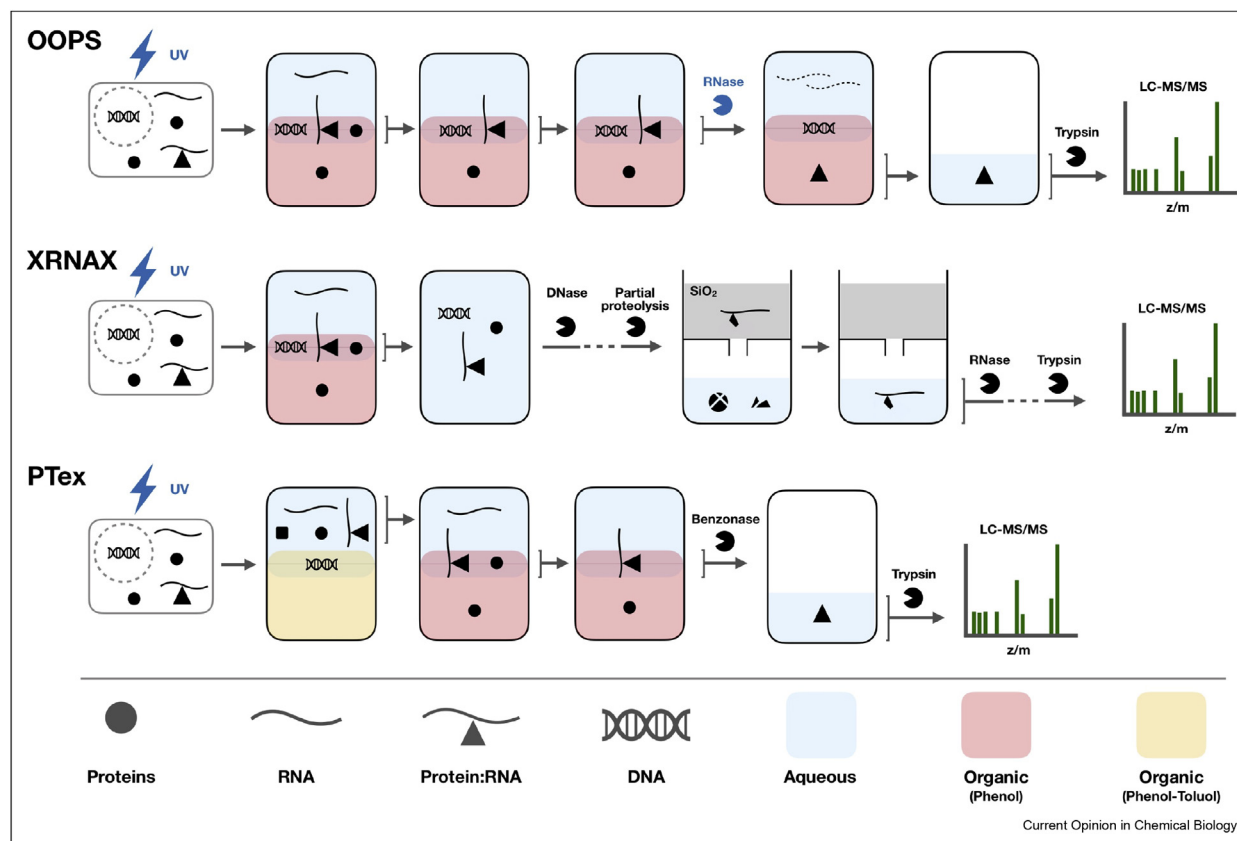
### Repurposing phase separation for comprehensive RBP recovery

A new exciting paradigm to recover the complete RBPome based on the inherent physicochemical properties of the RNA–protein complexes has recently emerged. This approach repurposes the classic biphasic aqueous and organic solvent–based sample partitioning to enrich RBPs independent of the sequence or length of bound RNA. Phenol phase separation–based techniques are a well-established approach to extract RNA and proteins, with RNA partitioned to the aqueous phase and protein to the organic phase [38–40]. Reasoning that UV-induced protein–RNA adducts would concentrate at the interface owing to opposing physical–chemical properties, three groups independently established methods to recover the enriched RBPs from the interface and process them for mass spectrometric analysis.

Although the repurposed phase separation enriches protein–RNA adducts at the interface, further steps are required to improve the enrichment of RBPs. Orthogonal organic phase separation (OOPS) [41], phenol toluol extraction (PTex) [42] and protein-crosslinked RNA eXtraction (XRNAX) [37], all use acidic phenol phase separation to obtain a crude protein–RNA adduct sample but apply very different approaches to reach the final protein–RNA extract (Figure 1).

OOPS is based on standard acid guanidinium thiocyanate-phenol-chloroform (AGPC; commercially available as TRIzol™, or equivalents). Improved enrichment of UV-crosslinked protein is achieved by three sequential rounds of AGPC phase separation with each round

Figure 1



**Phase separation-based approaches to enrich RNA-binding proteins.** Enrichment of protein–RNA using phase separations and differing approaches to remove unwanted macromolecules. Each method starts by UV CL to induce protein–RNA adducts and uses at least one round of acidic phenol phase separation to separate adducts from free protein and RNA. Methods diverge in how they obtain the final sample. The points at which controls can be performed are shown in blue. Only the significant steps in each protocol are shown.

increasing the enrichment of RBPs. Reliable RBP extraction is achieved through RNase treatment which releases the RNA-bound proteins into the final organic phase. This last step also helps to avoid any contaminants coenriched in the interface. XRNAX uses a single AGPC phase separation which is then resolubilized with sodium dodecyl sulfate. After DNA digestion, a partial protease digestion yields RNA:peptide adducts which are then amenable to further enrichment using silica-based columns to purify peptide-crosslinked RNA as a distinct entity. This approach was shown to significantly improve the overall enrichment of UV-crosslinked proteins relative to the first AGPC interface. Similarly, silica purification of peptide–RNA was used to identify RNA-binding sites within the OOPS workflow. PTex takes an alternative approach and uses an initial pH 7.0 phenol:toluol phase separation which partitions DNA and lipids to the interface and away from the protein and RNA in the aqueous phase. The aqueous phase is then recovered and subjected to two rounds of acidic phenol phase separation to enrich protein–RNA adducts at the interface and away from noncross-linked RNA and protein.

As with previous RBP capture approaches, all three phase separation methods use UV CL to form protein–RNA adducts but are theoretically agnostic to the technique used to generate the protein–RNA adducts. The addition of control samples that are not UV cross-linked enables a comparison of protein abundance in CL positive and negative samples and thus confident assignment of RNA interaction status. All three methods used RNA degradation (through either RNase or alkaline degradation) to establish that the proteins identified are RNA-dependent and OOPS incorporates this step into the workflow.

In comparison with oligo(dT)-based methods, phase separation approaches enrich RBPs independently of the poly(A) status of their cognate RNAs and possess significantly reduced input requirements, increasing their applicability. The self-contained nature of the sequential phase separation rounds enables OOPS and PTex to require the lowest sample amounts for RBP enrichment to date ( $\sim 3 \times 10^6$  and  $\sim 5 \times 10^6$  cells, respectively), with XRNAX requiring  $8 \times 10^7$  cells. As all of these approaches are poly(A)-independent, phase

separation methods are also compatible with bacteria. Thus, OOPS and PTex were used to obtain the first comprehensive RBPomes for *E. coli* and *Salmonella Typhimurium*, respectively [41,42]. In doing so, both identified not only canonical bacterial RBPs such as Hfq and ProQ but also putative novel RBPs, including *YihI*, *SipA* and *AhpC* in *S. Typhimurium*, which were validated using T4 polynucleotide kinase (PNK) assays. The simplicity of these three methods also makes them ideal to study dynamics in RNA-binding. XRNAX was applied to quantify changes in the RBPome after arsenite treatment, identifying a translation arrest, including the loss of RNA binding for the ribosomal subunits which bind mRNA in the 80S ribosome cleft [37]. Similarly, OOPS was used to quantify RBPome dynamics in response to nocodazole arrest and identified a coordinated increase in RNA binding for metabolic enzymes when the nocodazole-mediated inhibition of microtubule formation was removed [41].

The enriched protein–RNA adducts can also act as the starting point for downstream applications beyond the identification of RBPs and the quantification of their abundance. Of particular interest is the potential to identify the site of RNA-binding. Both OOPS and XRNAX publications included high-throughput identification of RNA-binding sites across the complete RBPome, yielding, for example, the first *in vivo* evidence for RNA interaction at GAPDH Rossmann fold and a novel RNA-binding domain, named WKF, in C7orf50. In addition, the enriched protein–RNA adducts can be used as the starting material for targeted approaches such as Western blotting to interrogate the RNA-binding of specific proteins, or CLIP-Seq–related approaches to detect specific RNA transcripts bound to a given protein of interest. In this case, the partial protease digestion step of XRNAX should be avoided.

### Limitations of phase separation methods

Despite the three approaches' shared aim to extract the complete RBPome, there are considerable differences downstream of the RBP extraction, including the mass spectrometers, peptide spectrum matching algorithms, protein quantification methods, and thresholds applied, which preclude a reasonable comparison between them based on the published datasets. An independent evaluation of the three methods for a typical experimental design(s) would be of immediate value to researchers in the protein–RNA interaction field. Regardless, all three methods have inherent limitations: any method relying on UV CL can retrieve nonphysiologically relevant protein–RNA interactions. The specific biological relevance of all RBPs recovered by these methods should therefore be further investigated. Moreover, RNA-specific cross-links have been shown to be strongly dependent on uracil over other nucleobases [43,44], which biases the recovery of RBPs to those containing

uracil in their binding site or nearby. In addition, phase separation methods are not suitable to interrogate the RNA-binding capability of glycoproteins because, being a protein conjugated to a carbohydrate, they share similar physicochemical properties as protein–RNA adducts.

### Concluding remarks and perspectives

In recent years, our understanding of RNA biology has changed substantially and many processes that were thought to be invariable, such as ribosome composition or tRNA availability, are now known to be fine-tuned [45,46]. RNA transcription, trafficking, and translation are still far from being a fully understood process, even for mRNA. Meanwhile, new regulatory and structural functions for lncRNAs continue to be discovered [47,48], and phase exclusion compartments are changing the way in which we understand cellular complexity [49,50]. To date, technical limitations have held back our ability to interrogate the protein–RNA interactions underpinning these processes from a system-wide perspective. Now, thanks to the development of organic phase separation methods to study RNA–protein interactions, we have a new, more systematic approach to address them. These methods represent more than a new tool to simply obtain comprehensive catalogs of RBPs. By facilitating the quantification of RNA-binding dynamics of RBPs, they open up the possibility of studying RBP behavior upon physiological and physiopathological perturbations, helping us to understand the role of RNA and RBPs in biological processes and pathologies. In particular, many RBPs have other known functions not related to RNA biology, and it will be interesting to explore if these combined capacities reveal novel interplays between biochemical or other regulatory functionalities. For instance, all three studies identified several DNA-binding proteins that also bind RNA, suggesting an additive or competitive function to bind to either type of polynucleotide. In addition, both OOPS and XRNAX showed that bromodomain-containing proteins were enriched in RNA-binding proteomes. Indeed, bromodomains, the structural motifs in chromatin readers which interact with acetylated lysines in histones, were recently shown to also serve as docking sites for eRNAs, leading to enhanced transcriptional cofactor activities [51]. Phase separation methods can be applied to any cellular model, facilitating the study of RNA–protein interactions from an evolutionary perspective. Furthermore, as techniques to enrich RNA–protein complexes, they are an extremely useful starting point for targeted methods CLIP-Seq–related methods [52–55] or RNA-centric ribonucleoprotein (RNP) complex pull downs [56,57]. Altogether, phase separation techniques represent an easy and affordable, yet comprehensive, flexible, and robust strategy to study RNA–protein interactions from an exciting new perspective.



## Conflict of interest statement

Nothing declared.

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